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1 **Species-specific Cd-detoxification mechanisms in lumbricid earthworms *Eisenia andrei*,**  
2 ***E. fetida* and their hybrids**

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23 **Abstract:**

24 Hermaphroditic lumbricid *Eisenia* sp. earthworms are ubiquitous and highly resistant to a  
25 variety of environmental stressors, including heavy metals. Among the progeny of laboratory  
26 mated inter-specific pairs of *Eisenia fetida* (*Ea*) and *Eisenia andrei* (*Ef*) there are fertile *Ha*  
27 hybrids derived from *Ea* ova fertilized by *Ef* spermatozoa and very rare sterile *Hf* hybrids  
28 from *Ef* ova fertilized by *Ea* spermatozoa. The aim of the first part of the experiment was to  
29 compare the life traits and whole body accumulation of cadmium in adult earthworms from  
30 genetically defined *Ea*, *Ef* and their hybrids (*Ha*) exposed for four weeks to commercial soil  
31 either unpolluted (control) or cadmium-spiked leading to moderate (M) or high (H) soil  
32 pollution (M=425 and H=835 mg kg<sup>-1</sup> dry soil weight). Such exposure impaired cocoon  
33 production but not affected earthworm viability despite the massive Cd bioaccumulation in  
34 the whole earthworm bodies reaching at M and H groups 316-454, 203-338, 114-253, and  
35 377-309 mg kg<sup>-1</sup> dry body weights of *Ea*, *Ef1*, *Ef2*, and *Ha*, respectively, surprisingly reaching  
36 maximum accumulation quantities in hybrids. The second part of the experiment aimed to  
37 investigate cadmium-related defense mechanisms at transcriptomic level in coelomocytes  
38 non-invasively extruded from coelomic cavities of the new sets of *Ea*, *Ef*, *Ha*, and *Hf*  
39 earthworms exposed to Cd in microcosms for 0 days (control), 2 days, and 7 days (M=425 mg  
40 kg<sup>-1</sup>). Expression level of stress-induced *Cd-metallothionein* (*mt*) and *superoxide dismutase*  
41 (*sod*) were gradually up-regulated, while the immune-connected lysenin (*lys*) was rapidly  
42 down-regulated; the expression of glutathione S-transferase (*gst*) and phytochelatin synthase  
43 (*pcs*) remained unaffected. *Mt* and *sod* gene up-regulation and *lys* gene down-regulation were  
44 especially pronounced in *Ea*-derived hybrids. In sum, capacity of cadmium bioaccumulation  
45 and detoxification mechanisms is more efficient in interspecific hybrids than in the pure *Ea*  
46 and *Ef* species.

47 **Keywords:** *Eisenia* sp.; hybrids; cadmium; Gene expression; detoxification; Bioaccumulation

48 **1. Introduction**

49 Cadmium (Cd) contamination of soils is a ubiquitous environmental problem that has resulted  
50 from uncontrolled industrialization, unsustainable urbanization, and intensive agricultural  
51 practices (Khan et al., 2017). The dispersion of cadmium in the environment had been  
52 increasing in the past few decades all over the world. As an example, currently, Cd  
53 contamination can be found as a direct result of most industrial activities such as, mining,  
54 smelting, transport, burning of fossil fuels, wastewater irrigation systems, and as a by-product  
55 of synthetic phosphate fertilizers (Grobela et al., 2019). Being a toxic element, Cd is a high  
56 threat to soil quality, food safety, and human health (Shahid et al., 2016). To date, numerous  
57 studies have investigated the toxic impact of Cd in plants and animals, including humans.  
58 Research focused on human exposure to cadmium shows that Cd induces several types of  
59 cancers, neurotoxic effects as well as kidney damage, bone demineralization, and the  
60 disruptions of the immune system (Buha et al., 2019). Overall, industrial activities involved in  
61 a large scale dispersal of heavy metals to the environment are associated with genotoxic and  
62 carcinogenic hazards, which creates a crucial challenge for regulatory authorities (Eom et al.,  
63 2017). Improvements in the technologies of contaminant detection and quantification in  
64 environmental samples are essential (Mahajan and Kaushal, 2018). Nevertheless, novel  
65 approaches that could take into account not only chemical hazards but its biological and  
66 ecological effects are currently needed for accurate safety evaluation (Khan et al., 2017).

67 Earthworms, living in close contact with soil, are constantly confronted with different  
68 pollutants, among the others high cadmium concentrations (Wang et al., 2019). Moreover,  
69 they are a crucial part of soil fauna, which helps to maintain soil structure and organic matter  
70 cycling via soil ingestion and excretion, making them key regulators of soil processes. Hence,  
71 earthworms are widely used as model organisms for the evaluation of the hazardous effects of  
72 different terrestrial toxicants (Yu et al., 2019).

73 Compared to other lumbricid species, composting earthworms from *Eisenia* genus are very  
74 resistant to most pollutants, including heavy metal contamination. Nevertheless, most of the  
75 early studies are focused on the *Eisenia* sp., not taking into account the distinction between *E.*  
76 *fetida* and *E. andrei* (Rombke et al., 2016, Suleiman et al., 2017). Proper distinction of the red  
77 earthworm *E. andrei* and striped *E. fetida* on basis of morphological criteria is sometimes  
78 unequivocal, thus shall be connected with species delimitation by the species-specific DNA  
79 sequences of the mitochondrial and/or nuclear genes. Such molecular markers revealed a  
80 clear distinction between *Ea* and *Ef* species, and at least two distinct mitochondrial lineages of  
81 the latter, described as *Ef1* and *Ef2* (e.g. Pérez-Losada et al. 2005; Otomo et al. 2008; Rombke  
82 et al. 2016). Combined analysis of sequences of the haploid mitochondrial genes (of the  
83 maternal origin) and diploid nuclear genes of maternal-paternal origin made possible to find  
84 out hybrids between laboratory-mated (*Ea+Ef1*) or (*Ea+Ef1*) pairs (Plytycz et al. 2018a,b;  
85 2020; Podolak et al. 2020).

86 Among the progeny of hermaphroditic *Ea+Ef* pairs appeared both specimens of the pure *Ea*  
87 or *Ef* species (derived by self-fertilization) and hybrids derived from *Ea* ova fertilized by *Ef*  
88 spermatozoa (called here *Ha*), but non *Hf* hybrids from *Ef* ova fertilized by *Ea* spermatozoa.  
89 These later appeared only among progeny of *Ha* hybrids mated with *Ef* pure species but were  
90 very rare and sterile. The *Ha* hybrids were relatively common and fertile, giving offspring  
91 with *Ea* species consisting off second (and then third) generations of *Ha* hybrids and pure *Ea*  
92 specimens (Plytycz et al. 2018a,b; Podolak et al. 2020), but fertility of hybrids was gradually  
93 diminished in the subsequent generations (Plytycz et al. 2020). The question arose whether  
94 hybrids are more sensitive to stressing factors than specimens of the pure parental species?

95 Our previous laboratory studies on effects of cadmium were performed on *E. fetida* (Brulle et  
96 al. 2008, Bernard et al. 2010) and more recently on *E. andrei* (Homa et al. 2015; Takacs et al.  
97 2016, Rorat et al. 2017). We have documented that exposure of *E. andrei* earthworms to soil

98 spiked with high concentration of cadmium chloride (500 mg kg<sup>-1</sup> air-dried soil), caused  
99 massive cadmium bioaccumulation in earthworm bodies without lethal effects; it caused  
100 inhibition of maturation of juveniles, inhibition of cocoon production by adults without  
101 adverse effects on regenerative processes (Takacs et al. 2016). Cadmium exposure even  
102 enhanced regeneration of amputated posterior segments that corresponded with induction of  
103 expression of metallothionein gene of this species (Rorat et al. 2017).

104 In our previous studies, the mechanisms of detoxification of Cd were investigated on  
105 transcriptomic level and some genes were identified as involved or potentially involved in  
106 those processes, namely, metallothionein (*mt*), glutathione S-transferase (*gst*), lysenin (*lys*),  
107 superoxide dismutase (*sod*), phytochelatin synthase (*pcs*) (Brulle et al. 2008, Bernard et al.  
108 2010, Homa et al. 2015). Among the others, it has been shown that the protective mechanisms  
109 were mainly based on a strong induction of a gene coding the cadmium metallothionein (*mt2*)  
110 in earthworms (Homa et al. 2015, Engelmann et al., 2016). The implication of the other genes  
111 mentioned before is not fully elucidated. Even if the two *Eisenia* species are very resistant to  
112 stressors and show similar responses overall, we noted specificities, in particular at the  
113 molecular level, which we decided to investigate in this work.

114 Therefore, it was reasonable to compare effects of cadmium exposure on *E. andrei*, two  
115 distinct lineages of *E. fetida* (*Ef1* and *Ef2*) and on hybrids appearing among progeny of *Ea+Ef*  
116 pairs mated in the laboratory settings (Plytycz et al. 2018a,b, 2020; Podolak et al. 2020). For  
117 this, the accumulation of Cd and the level of expression of genes directly or indirectly  
118 involved in the response to Cd were measured in pure species and their hybrids investigated at  
119 the same laboratory conditions.

120 The main goal of the first part of our experiments was to compare the life traits and the  
121 capacity of cadmium accumulation in whole bodies of adult earthworms from genetically  
122 defined *Ea*, *Ef1*, *Ef2* and their hybrids (*Ha*). Earthworms were exposed for four weeks to

123 commercial soil either unpolluted (control) or moderately or highly spiked with cadmium  
124 chloride at the same concentrations as that used during our previous studies (Takacs et al.  
125 2016; Rorat et al. 2017). The second part aimed for gaining insight into cadmium-related  
126 defense mechanisms at transcriptomic level in coelomocytes extruded from a new set of *Ea*,  
127 *Ef1*, *Ef2*, and *Ha/Hf* earthworms exposed for 0 days (control), 2 days or 7 days to moderately  
128 Cd-polluted soil in mesocosms. Overall, the upregulation of *mt* and *sod* genes and down-  
129 regulation of *lys* genes were more pronounced in hybrids than in parental species.

## 130 **2. Materials and methods**

### 131 **2.1. Earthworms**

132 Adult composting *E. andrei* (*Ea*) and *E. fetida* (*Ef*) earthworms deriving from laboratory  
133 stocks at the University in Lille (France) were cultured for a decade in the laboratory of the  
134 Institute of Zoology and Biomedical Research of the Jagiellonian University (Krakow,  
135 Poland), and in parallel for last four years in the laboratories of Rzeszow University (Poland).  
136 Earthworms were cultured in boxes with commercial soil at room temperature and fed *ad*  
137 *libitum* on boiled/dried tea, nettle and dandelion leaves. For several years some of them were  
138 used for studies on progeny of laboratory-paired genotyped virgin specimens giving in  
139 offspring specimens of *E. andrei* (*Ea*), two mitochondrial lineages of *E. fetida* (*Ef1* and *Ef2*)  
140 species, and interspecific hybrids (*H*) derived either from *Ea* ova (*Ha*) or *E1/Ef2* ova (*Hf*),  
141 each of them individually delimited by species-specific sequences of the mitochondrial COI  
142 gene and diploid nuclear 28S rRNA gene (Plytycz et al. 2018a; 2020; Podolak et al. 2020).

143 Some of these genetically delimited adult (clitellate) specimens were used for experiments  
144 performed in the same laboratory conditions (Plytycz et al. 2020; Podolak et al. 2020). One  
145 group of earthworms (54 individuals) was used for the measurement of cadmium

146 accumulation in the whole earthworm bodies, the second group (50 individuals) served as a  
147 source of coelomocytes for the measurement of the expression of stress-related genes.

## 148 **2.2. Soil preparation**

149 The air-dried commercial soil (PPUH BIOVITA, Tenczynek, Poland organic soil, 51.7%  
150 organic matter, pH=6.11) was spiked either with water (control) or with cadmium chloride  
151 (CHEMPUR, Piekary Slaskie, Polska), 500 mg kg<sup>-1</sup>, as described previously (Takacs et al.  
152 2016; Rorat et al. 2017). Control and Cd-polluted soils were kept separately or mixed 1:1,  
153 giving samples of unpolluted control soil, and those with high (425 ± 45.66 mg kg<sup>-1</sup>) or very  
154 high (835 ± 35.712 mg kg<sup>-1</sup>) cadmium concentration.

## 155 **2.3. Experimental design**

156 *Experiment 1 Cd accumulation in earthworms:* In total 54 earthworms were exposed either to  
157 unpolluted control soil or to soil with high cadmium concentration during 4 weeks in  
158 mesocosms. Briefly, four individuals from each group of species: *Ea*, *Ef1*, *Ef2* and *Ha* were  
159 placed in the plastic boxes (of 9 cm diameter) with perforated lids. At the end of the exposure  
160 period, the earthworms were depurated overnight in boxes with wet lignin substrate (Paso-  
161 Trading, Polska), and then sacrificed by freezing and prepared for the metal accumulation  
162 analysis.

163 *Experiment 2: Gene expression in Cd-exposed earthworms:* In total 50 individuals were used  
164 as a source of coelomocytes, i.e. the immune cells present in earthworm coelomic cavity, non-  
165 invasively retrieved from coelomic cavity by a mild electric shock as described in 2.4.3.1. *E.*  
166 *andrei*, *E. fetida*, and their *Ea*-derived (*Ha*) and *Ef*-derived hybrids (*Hf*) were exposed for 2  
167 and 7 days to highly polluted soil in mesocosms (boxes with with perforated lids, 9 cm  
168 diameter). The level of gene expression of glutathione S-transferase (*gst*), lysenin (*lys*),

169 metallothionein (*mt*), superoxide dismutase (*sod*) and phytochelatin synthase (*pcs*) was  
170 assessed in extruded coelomocytes, and related to the expression of two stable house-keeping  
171 genes – actin (*act*) and ribo13 (*r13*).

## 172 **2.4. Analyses**

### 173 **2.4.1 Earthworm genotyping**

174 Supravivally amputated tail tips of numerically coded adult earthworms were used for DNA  
175 extraction and genetic analysis using oligonucleotide primers amplifying species-specific  
176 variants of mitochondrial COI genes and nuclear 28S rRNA genes, as described earlier  
177 (Plytycz et al. 2018a,b; 2020; Podolak et al. 2020).

178 As previously established, mitochondrial species-specific sequences of the COI gene were  
179 called either ‘a’ or ‘f’/‘f2’ for *Ea* and *Ef1/Ef2*, respectively, while the diploid species-specific  
180 nuclear markers of the 28S rRNA gene were called either ‘A’ or ‘F’ for *Ea* and *Ef*,  
181 respectively. In a results, the investigated specimens were genotyped either as pure species,  
182 *Ea* (*aAA*), *Ef1* (*fFF*), *Ef2* (*f2FF*), or inter-specific hybrids, either *Ea*-derived *aAF*, *Ef*-derived  
183 *fFA* with the two first letters (*aA*-, *fF*-, *f2F*) representing ova-derived genes, i.e. maternity,  
184 and the last letter (*A* or *F*) representing spermatozoa-derived genes, i.e. paternity.

### 185 **2.4.2. Cadmium content**

186 Cadmium content in soil and in whole earthworm bodies was measured by slightly modified  
187 methods used in our previous studies (Takacs et al. 2016; Rorat et al. 2017). Soil samples  
188 were dried at 105°C for 24 h and then divided into subsamples about 0.5 g of dry soil mass of  
189 each. Cadmium concentration in soil was determined after half open wet digestion in a quartz  
190 glass with 10 mL of mixture of concentrated HNO<sub>3</sub> (69.0-70.0% to trace metal analysis,  
191 Baker Instra-Analyzed) and HClO<sub>4</sub> (70% a.r, Chem-Lab) (7:1 v/v), using hot plates.

192 Cadmium concentrations were measured by atomic absorption spectrometry (AAS) with a  
193 flame and atomic absorption spectrometry with a graphite furnace nebulizer (Perkin-Elmer) to  
194 achieve the desired method sensitivity. Accuracy was verified by analyzing four blanks and  
195 three replicates of standard certified material (Loamy Sand 10 CRM033-50G) with the  
196 samples.

197 Prior to the analysis of cadmium concentration in whole earthworm bodies, earthworms were  
198 kept for 2 days on wet filter papers to remove their gut content, frozen, and then dried in  
199 vacuum drier in 70°C for 24 h to achieve constant dry mass. For determination of cadmium  
200 concentration in earthworm bodies, the wet digestion method was applied but to reach the  
201 required ratio 1: 20 only 4 mL of concentrated HNO<sub>3</sub> (69.0-70.0% to trace metal analysis,  
202 Baker Instra-Analyzed) were used. Cadmium concentrations in earthworm bodies were  
203 measured by atomic absorption spectrometry methods with four blanks and three replicates of  
204 standard certified material (Bovine Liver SRM 1577c) with the sample in digestion.

205 The cadmium concentrations were calculated on a dry mass of each sample of soil or  
206 earthworms and are presented in mg kg<sup>-1</sup> (µg g<sup>-1</sup>).

207

### 208 **2.4.3. Gene expression analysis**

#### 209 2.4.3.1. Extrusion of coelomocytes extrusion via electrostimulation

210 After 24 h depuration on wet lignin (Paso-Trading, Poland), earthworms were placed  
211 individually on petri dishes and stimulated for 30 s with a 4.5 V electric current to expel  
212 coelomic fluid with suspended coelomocytes through the dorsal pores (Homa et al. 2015;  
213 Rorat et al. 2017). Pellets of coelomocyte samples were resuspended in 2 mL of TriReagent  
214 (Molecular Research Center Inc., USA) for RNA extraction.

#### 215 2.4.3.2. Extraction of RNA and reverse transcription

216 Expression of five target and two house-keeping genes (HKG) was performed on the total  
217 RNA extracted from coelomocytes by TRI Reagent® (Molecular Research Center, USA),  
218 according to manufacturer's instructions. Overall, coelomocytes' pellets from each sample  
219 were suspended in 2 mL of Tri Reagent and 1 mL was later used for each extraction. Samples  
220 were then treated with DNase I, RNase-free (1 U/μL) (Thermo Fisher Scientific, USA)  
221 according to manufacturer's instructions. The quantity and quality of isolated RNA were  
222 analyzed spectrophotometrically using spectrometer with a LVis nano plate (SPECTROstar  
223 Nano, BMG LABTECH, USA, LVis Plate, BMG LABTECH, USA). RNA integrity was  
224 confirmed on 2% agarose gel stained with ethidium bromide (Ethidium Bromide Solution (10  
225 mg mL<sup>-1</sup>), Thermo Scientific, USA). The amount of isolated RNA allowed us to perform the  
226 reverse transcription of 1.5μg cDNA using QIAGEN OMNISCRIPT RT KIT (QIAGEN,  
227 Germany), according to manufacturer instructions. cDNA samples were then stored in -20°C  
228 until further analyses.

#### 229 2.4.3.3. Real-time qPCR amplification.

230 The mRNA levels of chosen genes were determined by real-time quantitative PCR after  
231 reverse transcription. For qPCR, Agilent Mx3000P QPCR System (Agilent Technologies,  
232 USA) with 2X Takyon for SYBR Assay - No ROXqPCR Kits for SYBR Assay (Eurogentec,  
233 Belgium) were used, and reactions were performed on 96-well qPCR plates (Eppendorf  
234 twin.tec® PCR Plates, Eppendorf AG, Germany). All qPCR reactions were performed as  
235 follows: 95°C 5 min and 40 cycles at 95°C 30 s, 55°C 30 s, and 72°C 60 s. After 40 cycles,  
236 the last step of the program consisted of 72°C for 5 min. The *Eisenia* sp. specific primes were  
237 used for actin (*act*), ribosomal protein S13 (*r13*), metallothionein (*mt*), glutathione S-  
238 transferase (*gst*), phytochelatin synthase (*pcs*), lysenin (*lys*) and superoxide dismutase (*sod*)  
239 (Brulle et al., 2008, Homa et al. 2015) after the validation of their compatibility with hybrids.  
240 The selected primers are presented in Table 1. Expression levels were determined as the

241 number of cycles needed for the amplification to reach a threshold fixed in the exponential  
242 phase of PCR reaction (Ct). Levels of expression and amplification effectiveness of target  
243 genes were compared to the expression of two constitutively expressed and validated house-  
244 keeping genes. qPCR efficiency of each target gene was calculated according to the method  
245 previously described (Chaâbene et al., 2017). Overall, the relative expression level was  
246 calculated according to the following formula:

247 
$$R = 2^{\Delta Ct(\text{target gene}) - \Delta Ct(\text{reference gene})}$$

248

249 Table 1. qPCR primer sequences

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size (bp)	Accession number
<i>r13</i>	5'CGCACGGTTTTAGTTTCT3'	5'CCATGCGAGTCTCGAAG3'	148	MG076964
<i>act</i>	5'CCATCCATCGTCCACAG 3'	5'GCATGTGTGAGTCCTAATT3'	147	EY893028.1
<i>mt</i>	5'AAAGTGAGTGCTTGCC3'	5'ACTGATGACAGAGTTCCG3'	120	KP770991.1
<i>gst</i>	5'TGGAAGTGGAGGTTTCCTTG3'	5'TGAGGTGGCCAAGTCTTTTC3'	161	EY892760.1
<i>pcs</i>	5'TCATGGTCCTGAACACG3'	5' CACAAGTTGCCGAAACTC 3'	154	KP770990.1
<i>lys</i>	5'CGGCAACAAACGTCTAC3'	5'GTGAAATACAGGCAGAAGC 3'	163	MG076962.1
<i>sod</i>	5'GGTGCTCACTTCAACCCATT3'	5'AAGATCGTCCACCAGCTCAT3'	167	DQ286712.1

250

## 251 2.5. Data analysis

252 HKG stability was quantified using two software's: BestKeeper and NormFinder. All results  
 253 are expressed as means  $\pm$  standard deviation, n=3-6. Descriptive statistics and statistical  
 254 analyses were produced using the OriginPro 2015 software and Statistica. Different letters  
 255 "a", "b" "c", "d" on top of bars indicate statistically significant differences according to the 1  
 256 one-way analysis of variance.

## 257 3. Results

### 258 3.1. Genetic species delimitation

259 Cytoplasm-rich ova with plenty mitochondria are fertilized by spermatozoa with very scarce  
 260 cytoplasm and a few mitochondria. DNA barcoding based on specific sequences of  
 261 mitochondrial haploid COI gene shows three distinct branches; 'a' for *Ea* or 'f'/'f2' for two

262 mitochondrial lineages of *Ef*, namely *Ef1* and *Ef2* (Plytycz et al. 2020). Species-specific  
263 maternal-paternal diploid nuclear sequences of 28S rRNA gene, are either 'AA' (for *Ea*) or  
264 'FF' for *Ef* or 'AF' for interspecific hybrids. Fertilization of 'aA' ovum by 'A' spermatozoon  
265 gives aAA zygote of the pure *Ea* species, while 'aA' ovum fertilized by 'F' spermatozoon  
266 gives *Ea*-derived aAF hybrid. The 'fF' or 'f2F' ova fertilized by 'A' spermatozoa give fFA or  
267 f2FA hybrids, respectively (Figure 1). All sequences are deposited in GenBank under the  
268 following accession numbers: MT646836-MT646899 and MT649303-MT649399. All the  
269 earthworms survived the experiment but cadmium exposure inhibited cocoon production  
270 almost completely (data not shown).

### 271 **3.2. Cadmium content in soil and in whole earthworm bodies**

272 The cadmium concentration in the commercial unpolluted soil was very low (L;  $0.11 \pm 0.107$   
273  $\text{mg kg}^{-1}$ ); in soil spiked with cadmium chloride was very high (H;  $835 \pm 35.712 \text{ mg kg}^{-1}$  dry  
274 weight) and intermediate in 1:1 mixture of these soil samples (M;  $425 \pm 45.66 \text{ mg kg}^{-1}$  dry  
275 weight) (Table 2). Cadmium concentrations in earthworms living in unpolluted soil were low  
276 ( $0.69 - 1.12 \text{ mg kg}^{-1}$ ) and did not differ significantly between *Ea*, *Ef1*, *Ef2*, and *Ha* groups of  
277 earthworms.

278 Cadmium concentrations in whole bodies of earthworms kept in soil with M or H cadmium  
279 pollution were overlapping ( $113.79 - 376.73$  and  $252.68 - 454.18 \text{ mg kg}^{-1}$ , respectively), with  
280 lowest accumulation capacity in *Ef2* earthworms ( $113.79$  and  $252.68 \text{ mg kg}^{-1}$  in M and H soil,  
281 respectively), but these values were not significantly different from those in the second  
282 lineage of the *Ef* species, i.e. *Ef1* ( $203.43$  and  $337.66$  respectively). The whole body Cd  
283 bioaccumulation in *Ea* reached  $316.43 \text{ mg kg}^{-1}$  in the M soil and was significantly higher in  
284 the *Ea* from the H soil ( $454.19 \text{ mg kg}^{-1}$ ). Cadmium bioaccumulation was most efficient in the  
285 *Ha* hybrids reaching plateau already in the M soil, as the values at M and H soil were similar  
286 ( $376.73$  and  $309.246 \text{ mg kg}^{-1}$ , respectively).

287 Table 2. Concentration of Cd in soil and earthworms' bodies after 4-week exposure in  
 288 unpolluted (L), intermediate (M) or very high (H) soil. Results shown as means  $\pm$  SD, n=3 - 6.  
 289 Mean concentrations in earthworm bodies not sharing the same small letters (in columns) or  
 290 capital letters (in rows) are statistically significantly different according to ANOVA with post  
 291 hoc Tukey's test.  
 292

Groups	Cd concentration in soil and earthworm bodies [mg kg <sup>-1</sup> ]			
	L	M	H	
<b>Soil</b>	0.11 $\pm$ 0.107	425 $\pm$ 45.66	835 $\pm$ 35.712	
<i>Earthworms</i>	<i>Ea</i>	1.09 $\pm$ 0.38 aA	316.43 $\pm$ 33.09 bB	454.19 $\pm$ 37.58 bC
	<i>Ef1</i>	0.76 $\pm$ 0.26 aA	203.43 $\pm$ 82.7 abB	337.66 $\pm$ 115.12 abB
	<i>Ef2</i>	0.69 $\pm$ 0.15 aA	113.79 $\pm$ 28.16 aB	252.68 $\pm$ 62.59 aC
	<i>Ha</i>	1.12 $\pm$ 0.10 aA	376.73 $\pm$ 136.99 bB	309.23 $\pm$ 59.50 abB

### 293 3.3. Gene expression in earthworms

#### 294 3.3.1. Reference genes

295 For determination of the stability of chosen housekeeping genes, the NormFinder and  
 296 BestKeeper software were used. Overall, the expression of both chosen reference genes was  
 297 stable across all experimental conditions and species (Appendix 1).  
 298

#### 299 3.3.2. Expression of *gst* and *pcs* genes

300 The expression level of *pcs* and *gst* was undetectable in coelomocytes of *E. andrei* in both  
 301 clean and Cd-contaminated soil. However, a negligible expression of those genes was  
 302 observed for *Ef1*, *Ef3* and *Ha* exposed to soil contaminated by cadmium (Figures 2A and 2B).

### 303 3.3.3 Expression of *mt*, *sod*, and *lys* genes

304 Only two rare *Ef*-drived hybrids (*Hf*\*) were used for analysis of *mt*, *sod*, and *lys* expression. In  
305 unpolluted soil (i.e. on day 0 of cadmium exposure), the expression of *mt* and *sod* was very  
306 low while it was relatively in a case of *lys*. In each instance, it was similar to the expression of  
307 these genes in other specimens from unpolluted soil, i.e. on day 0 of cadmium exposure (Fig.  
308 3, right columns of rows A, B, and C).

309 In all tested organisms, a significant induction of the expression of *mt* and *sod* was observed  
310 after exposure to cadmium (Figure 4, rows A and B), while expression of *lys* was drastically  
311 diminished (Fig. 3, row C).

#### 312 3.3.3.1 Expression of *mt*

313 The expression rate of metallothionein was negligible in earthworms exposed to clean,  
314 uncontaminated soil. In coelomocytes of *Ef1*, *Ef2* and *Ha*, the expression of *mt* was  
315 significantly ( $p < 0.05$ ) induced after already two days of exposure, and in *Ef1* and *Ha*  
316 continued to increase further until the 7-day timepoint. However, in coelomocytes of *E.*  
317 *andrei*, the induction of *mt* expression occurred later than for other earthworms, and was  
318 significant only after 7 days post exposure (Figure 3, row A, left column). At each time point  
319 the interspecies differences were statistically insignificant (Fig. 3, row A, right column).

#### 320 3.3.3.2 Expression of *sod*

321 Similar results were observed for the expression rate of *sod* (Figure 3, row B). Once again, the  
322 expression rate for all organisms living in clean soil was almost undetectable while the  
323 exposure to cadmium caused a significant increase in *sod* expression. However, two days  
324 post-exposure, the expression rate of *sod* in *E. andrei* was still almost no detectable, whereas  
325 for all other organisms was already significantly ( $p < 0.05$ ) induced. At day 7 day after  
326 cadmium exposure, expression of *sod* significantly increased versus day 2 in *Ea*, *Ef2*, and *Ha*

327 (Fig. 3, row B, left column). The interspecies differences were insignificant on day 0 and day  
328 2, while on day 7 was significantly higher in *Ha* than in *Ea*, *Ef1*, and *Ef2* (Fig. 3, row B, right  
329 column).

### 330 3.3.3.3 Expression of *lys*

331 The expression of lysenin gene was highly influenced by exposure to different soil conditions  
332 across all tested organisms. In all cases, its expression was at its highest in coelomocytes of  
333 earthworms exposed to clean soil and the Cd contamination caused a severe and significant  
334 ( $p < 0.05$ ) downregulation of that expression (Fig. 3, row C). Moreover, for *Ef2* and *Ha*, the  
335 downregulation was significant for both, 2 and 7 day post exposure, whereas for *Ea* and *Ef1* it  
336 was downregulated by 2 days post-exposure, and then the expression rate stabilized and  
337 stayed at the same level without further downregulation (Figure 3, row C, left column).  
338 Interspecies comparisons revealed the lack of statistically significant differences in day zero  
339 and day 2 after cadmium exposure while on day 7 *lys* expression differed significantly  
340 between species, being the highest in *Ef1*, and then in *Ea*, *Ef2* and *Ha* (Fig. 3, row C, right  
341 part).

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#### 350 4. Discussion

351 Adult specimens of hermaphroditic lumbricid earthworms *E. andrei* and *E. fetida*, the latter  
352 with two distinct lineages *Ef1* and *Ef2*, and interspecific Ha hybrids developed from *Ea* ova  
353 fertilized by *Ef* spermatozoa were fully viable during four week exposure to cadmium-  
354 polluted soil ( $425 \pm 45.66 \text{ mg kg}^{-1}$  or  $835 \pm 35.712 \text{ mg kg}^{-1}$ ) despite a high cadmium  
355 accumulation in the whole earthworm bodies. Bioaccumulation was dose-dependent, similar  
356 in *Ef1/Ef2* lineages of *Ef*, and lower than that in *Ea*. In contrast, *Ea*-derived hybrids reached  
357 plateau of Cd-accumulation capacity already at lower cadmium concentration in soil.  
358 Hypothetically, all investigated specimens from genus *Eisenia* are able to survive in Cd-  
359 contaminated environments due to adapting the same or similar detoxification mechanisms to  
360 those described previously at transcriptomic level in representatives of *E. andrei* and *E. fetida*  
361 (Homa et al. 2015, Rorat et al. 2017), while data concerning hybrids were vastly unexplored.  
362 Therefore comparative studies on the expression of genes involved in Cd detoxification were  
363 imperative. Second part of previous investigations revealed that the expression of stress-  
364 related genes upon exposure to Cd-polluted soil at  $425 \pm 45.66 \text{ mg kg}^{-1}$  was either up-  
365 regulated (*mt* and *sod*), down-regulated (*lys*) or unchanged (*gst* and *psc*).

366

#### 367 *Expression of genes for metallothionein and phytochelatin synthase*

368 In a study by Brulle et al. (2008) performed on *E. fetida*, the induction pattern of genes  
369 encoding phytochelatin synthase was significantly increased in earthworms exposed to a low  
370 concentration of cadmium ( $8 \text{ mg kg}^{-1}$ ). However, in higher concentrations, no difference was  
371 noticed between Cd-exposed earthworms and earthworms not exposed to any contamination,  
372 which suggests that *pcs* expression is only induced during low exposure to cadmium.  
373 Similarly to our study, expression of metallothionein was highly up-regulated after Cd  
374 exposure, and in the mentioned Brulle et al. (2008) study, it was also shown to be dose-

375 dependent in high Cd concentrations. Such relation between different responses of the  
376 expression of *pcs* and *mt* genes is of interest when considering their role in metal  
377 detoxification in animals. In low concentrations of Cd, the expression of *pcs* was shown to be  
378 significant and responsible for metal chelation in worm cells (probably via the synthesis of  
379 Phytochelatins (PCs) although the presence of PCs has not been demonstrated), whereas such  
380 induction was only noticed at a small exposure level. This is on the contrary to the effects of  
381 high concentrations of Cd, where similarly to our study, *pcs* expression patterns did not show  
382 any change of their level over control animals, whereas the expression rate of *mt* was  
383 substantially upregulated. In another study, focused on *Caenorhabditis elegans*, the  
384 toxicogenomic analysis after exposure to cadmium also showed no effect on the expression of  
385 *pcs-1* gene (Cui et al., 2007). Moreover, a recent transcriptomic study on the same species: *C.*  
386 *elegans*, found similar results – after exposure of nematods to cadmium (300µM) and zinc  
387 (500µM), the expression change of *pcs-1* was determined via qPCR followed by  
388 normalization by the invariant ribosomal *rla-1*. The results showed, that the *pcs* response to  
389 Zn and Cd contamination was at best negligible, and the change in comparison to control  
390 animals was not significant ( $p < 0.05$ ) (Essig et al., 2016). By combining these studies, we can  
391 see that phytochelatins and metallothioneins possibly act together to protect the cells against  
392 Cd. Phytochelatins have more of an immediate role at lower concentrations and  
393 metallothioneins coming later with higher induction. In addition, a comparison between the  
394 chosen gene expression profiles in earthworms exposed or unexposed to Cd showed up-  
395 regulation of two genes for all tested earthworms after Cd exposure – metallothionein and  
396 superoxide dismutase as well as down-regulation of lysenin. Such alterations between the  
397 expressions of those genes suggest that earthworms can oppose and reduce the toxic effects of  
398 Cd through several different pathways. Moreover, in the presented study, we noticed that the  
399 *mt* overexpression due to Cd exposure occurs not only in *E. fetida* but also in *E. andrei* and

400 hybrids between those species, which suggest, that this biomarker is suitable for both species.  
401 However, results suggest that *E. andrei* (*aAA*) activate its detoxification mechanisms slower  
402 than *E. fetida* (*fFF*) and their hybrids (*aAF*). Moreover, fast up regulation of expression of *mt*  
403 genes in hybrids suggests, that they obtained the nuclear allele responsible for a fast reaction  
404 from *E. fetida*, which showed similar tendencies, and not from *E. andrei*. In addition, such  
405 results could indicate that perhaps, *Ef* earthworms possess another detoxification mechanism  
406 or a specific regulation mechanism that is responsible for faster *mt* expression in comparison  
407 to *Ea*. Thus, by cloning of the genes coding *mt* in both species and studying of the regulation  
408 of the gene is needed to broaden our understanding of such response to metal exposure.  
409 Similar to our study, the induction of overexpression of the *mt* gene was recently shown in *E.*  
410 *andrei* earthworms after exposure to copper, which is a widely used fungicide, especially in  
411 organic farming (Mincarelli et al., 2019). Moreover, an induction of *mt* expression was also  
412 noticed in other earthworms including *Dendrobaena octaedra* and *Lumbricus rubellus*  
413 exposed to Cu (Mustonen et al., 2014, Höckner et al., 2015). The population of *Dendrobaena*  
414 *octaedra* exposed to Cu-Ni contaminated soil was shown to have higher *mt* expression levels  
415 in comparison to earthworms not exposed to metals. What is even more interesting, during  
416 artificial exposure experiment, exposure affected the *mt* expression, but only in the  
417 earthworms collected from the clean, unpolluted soil, showing that there is a delay in the *mt*  
418 response of earthworms which were not exposed to metals in the past. On the contrary,  
419 earthworms collected from the Cu-Ni smelting site showed not only higher but also constant  
420 levels of *mt* expression (Mustonen et al., 2014). Such results reflect on the role of the *mt*  
421 expression and the homeostasis of several metals and protection against oxidant damage.  
422  
423 *Superoxide dismutase and Glutathione S-transferase*

424 Glutathione S-transferase (GST) is an enzyme known to take part in the metabolism of  
425 lipophilic organic contaminants and in cellular protection against oxidative stress. A study by  
426 Mkhinini et al. (2019) showed an induction of *gst* expression after the exposure of *E. andrei*  
427 to treated wastewater (TWW) containing several trace metals. However, such effects were  
428 observed in earthworms exposed to TWW for more than 20 days. Our study showed no effect  
429 of cadmium exposure to the expression of this gene in a 7-day period, which could suggest  
430 that the *gst* mechanism is induced after prolonged, chronic exposure to heavy metals, but it is  
431 not an immediate response to the presence of contamination. In another study, the antioxidant  
432 gene expression of *E. fetida* was explored after worm exposure to various concentrations of  
433 hexabromocyclododecane. The results showed a significant up-regulation of the *sod* gene but  
434 no significant differences in the expression rate of *gst*, which further suggests its involvement  
435 in protection against the oxidative damage occurs during a prolonged period of time (Shi et  
436 al., 2018). Moreover, a recent study of Maity et al. (2018) focused on the oxidative stress  
437 response of *E. fetida* and *Eityphoeus waltoni* exposed to Cd, the overexpression of the *gst*  
438 gene increased significantly post-exposure and was dose-dependent. However, in *E. fetida*,  
439 after 2 days post-exposure, the expression of *gst* remained the same as in control animals,  
440 which also suggest its late-onset. In a transcriptomic study by Chai et al. (2019), focused on  
441 cadmium toxicity in *E. fetida*, a significant up-regulation of *sod* genes was also observed in  
442 earthworms exposed to 10, 30, 60 mg/kg of Cd<sup>2+</sup> in dry soil, respectively.

443

#### 444 *Lysenin*

445 Lysenin is a protein involved in immunity processes produced by coelomocytes and  
446 chloragocytes (Munguira et al., 2017). The presence of lysenin and lysenin-related proteins  
447 was documented in *E. andrei* and *E. fetida* and their putative hybrids (Swiderska et al. 2016).  
448 In the present study, the expression rate of *lys* gene was significantly down-regulated post Cd

449 exposure in all tested animals (*Ea*, *Ef*, *Ha*). A similar decrease in *lys* expression was noticed  
450 by Rorat et al. (2017) in *E. andrei* and Brulle et al. (2011) in *E. fetida*. However, the  
451 mechanisms behind such response are still unknown. In a study by Wang et al. (2010) the  
452 expression of *lys* was also shown to diminish after earthworms were exposed to *E. coli*.  
453 Besides, Wu et al. (2013), discovered that lysenin-related protein was significantly up-  
454 regulated post-exposure to PAH phenanthrene. Hence, lysenin expression in earthworms can  
455 have a complex response to exposure to different contaminants. Overall, the results imply the  
456 existence of a complex regulatory network that needs to be further explored in future studies.

457

#### 458 *Hybrids response to environmental stressors*

459 We have shown previously that *Eisenia andrei* (*Ea*) and *E. fetida* (*Ef*) of French provenance  
460 are capable of hybridization, as viable offspring appeared among progeny of laboratory-mated  
461 inter-specific *Ea+Ef* pairs. Recently we have shown that hybridization is not unique for  
462 French *Ea/Ef* earthworms, but is shared by laboratory-mated earthworms from French,  
463 Hungarian, and Polish laboratory cultures (Podolak et al. 2020; Plytycz et al. 2020), but  
464 fertility of hybrids is gradually diminished in the subsequent generations (Plytycz et al. 2020).  
465 Inter-specific hybrids were detected in natural populations of *Ea* and *Ef* from Scandinavia  
466 (Martinsson and Erseus, 2018) thus further data on hybridization in natural environment, even  
467 such highly polluted, including compost heaps, are expected.

468 Both *E. andrei* and *E. fetida* earthworms as well as their hybrids are perfect cadmium  
469 bioaccumulators and can survive in heavily contaminated soil due to efficient protective  
470 mechanisms. *Mt* and *sod* upregulation and *lys* down-regulation are especially pronounced in  
471 hybrids. Therefore we shall exclude such a possibility that the improper functioning of  
472 investigated stress-related genes is the main factor responsible for gradual impairment of  
473 hybrid fertility in the subsequent generations (Plytycz et al. 2020).

474 Interestingly, similar to our results, recent research on several species of plants indicated that  
475 hybrids can outperform their parental species after exposure to different stressors (both biotic  
476 and abiotic). As an example, a couple of types of poplar hybrids had been shown to better  
477 adapt to suboptimal temperatures and demonstrate superior growth, particularly under the less  
478 favorable, suboptimal temperature conditions (Guet et al., 2015). Overall, the interspecific  
479 hybridization has recently become of-interest to forest geneticists for tree improvement by  
480 assessing the possible feasibility of transferring desirable traits such as higher resistance to  
481 contamination, droughts, or diseases, among species (Juranović-Cindrić et al., 2018). Apart  
482 from trees, another study showed a variation between Cd uptake in maize hybrids, which can  
483 be used for phytoremediation of Cd-contaminated soil as well as for the selection and  
484 breeding of new, Cd-tolerant genotypes of maize (Akhtar et al., 2016, Wang et al., 2015).  
485 Similar tendencies had been observed for the accumulation of Cr in sunflower hybrids, which  
486 is currently used in the selection and breeding of Cr-tolerant genotypes of sunflowers (Farid et  
487 al., 2017). However, the molecular basis of such differential accumulation and the differences  
488 in the genetic makeup of both hybrids and parent species are scarcely researched and need to  
489 be further investigated in both, plants and earthworms exposed to contamination (Wang et al.,  
490 2015, Naeem et al., 2016, Juranović-Cindrić et al., 2018).

491

## 492 **5. Conclusions**

493 The insights gained in the presented study have improved our understanding of metal  
494 resistance of *E. fetida*, *E. andrei* and their hybrids. The results indicate that all of them are  
495 very efficient cadmium bio-accumulators with similar mechanisms of Cd detoxication.  
496 Consistently, upregulation of *mt* and *sod* expression was fastest in hybrids, slower in *Ef* and  
497 slowest in *Ea*. Hypothetically *Ef* and *Ea* differ in their mechanisms controlling expression of

498 stress-related genes and immunity-connected genes thus further studies of these genes and  
499 their interactions in hybrids are necessary.

500

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662

663 **Figure Captions**

664 Figure 1. Maximum-likelihood phylogram for the species-specific sequences ‘a’ or ‘f’/‘f2’ of  
665 the mitochondrial COI gene, with additional information on the ‘A’ or ‘F’ nuclear sequences  
666 of 28S rRNA nuclear gene of the same individuals with the same code, showing the pure aAA  
667 (*Ea*) and fFF/f2FF (*Ef1/Ef2*) species and their aAF or f2FA hybrids.

668 Figure 2. Relative expression level of *gst* (A) and *pcs* (B) in coelomocytes of groups of  
669 earthworms exposed to cadmium polluted soil concentration for 0 days (control), 2 days, or 7  
670 days: Effects of time of exposure. Different letters “a”, “b” “c”, “d” on top of bars indicate  
671 statistically significant differences according to the Kruskal–Wallis one-way analysis of  
672 variance ( $p < 0.05$ ).

673 Figure 3. Relative expression level of *mt* (row A), *sod* (row B) and *lys* (row C) in  
674 coelomocytes of groups of earthworms exposed to cadmium polluted soil concentration for 0  
675 days (control), 2 days, or 7 days; Left column - effects of time of exposure; right column  
676 interspecies comparisons between *Ea*, *Ef1*, *Ef2*, and *Ha* earthworms at particular time points.  
677 Results shown as means  $\pm$  SD,  $n=6$ ; *Hf\** - mean from two specimens only. Different letters  
678 “a”, “b” “c”, “d” on top of bars indicate statistically significant differences according to the  
679 Kruskal–Wallis one-way analysis of variance ( $p < 0.05$ ).

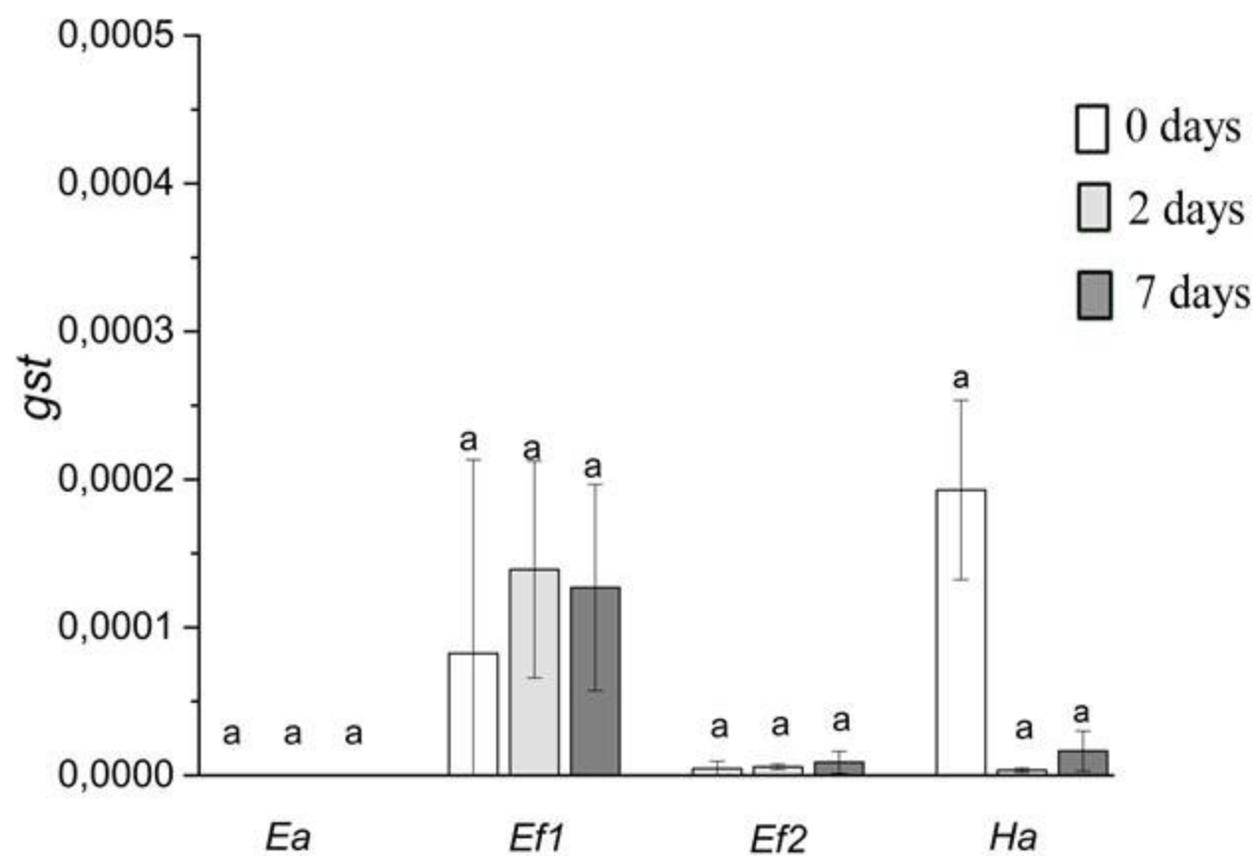
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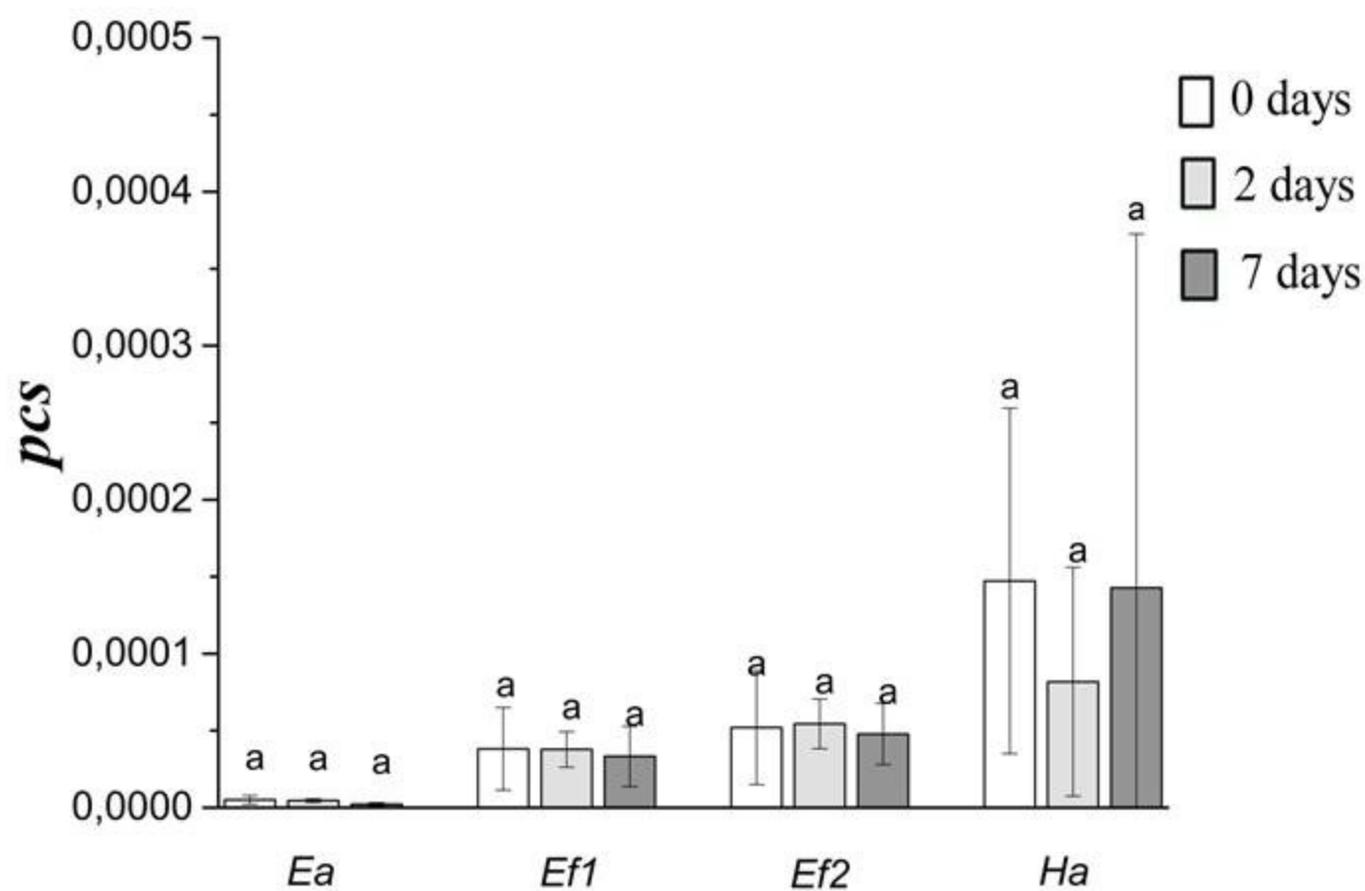
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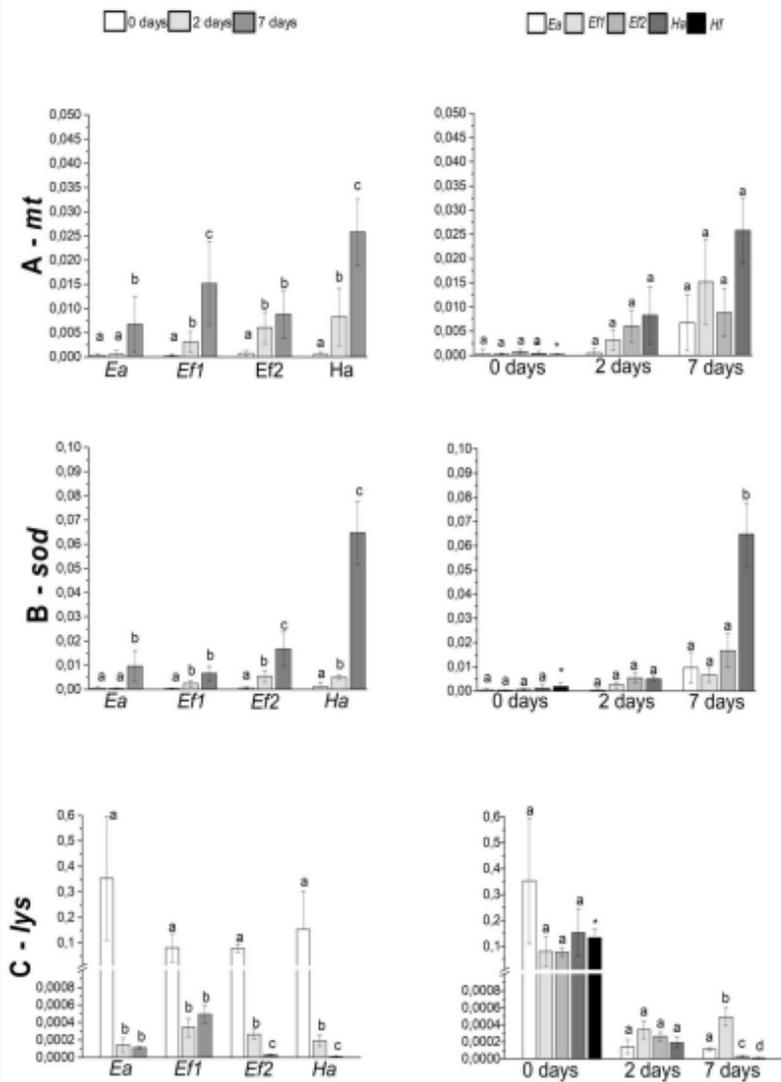
A)



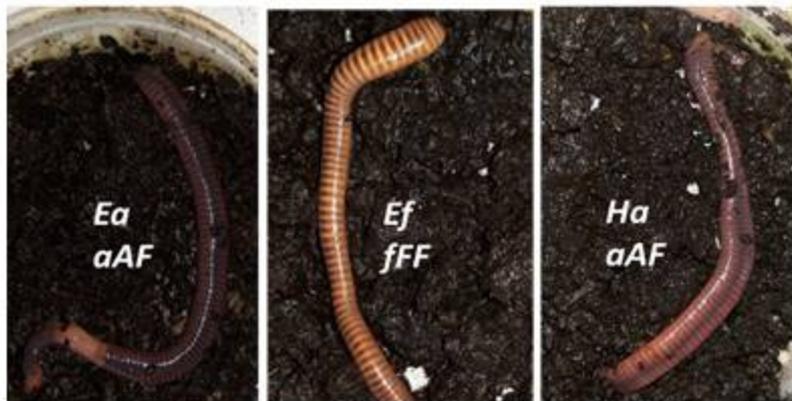
B)



# Relative expression level



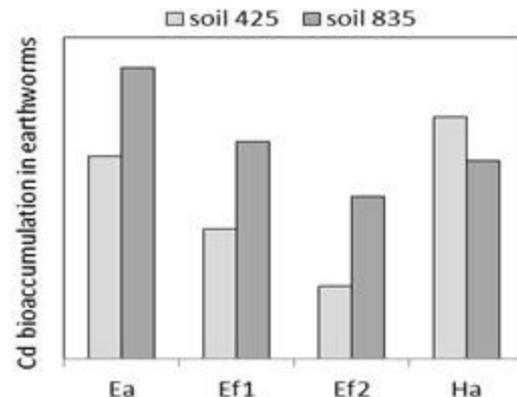
Exposure on Cd  
(425 or 835  
mg/kg)



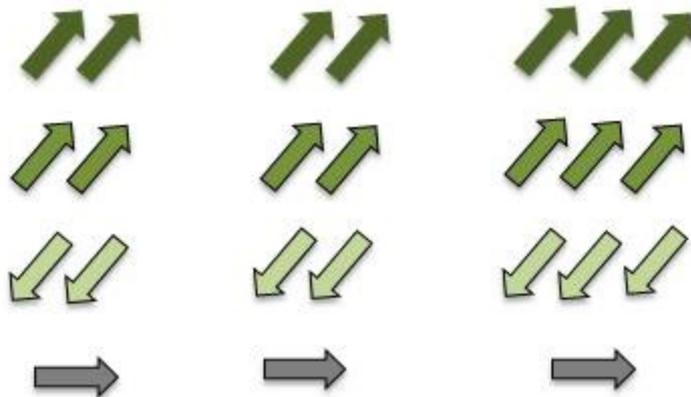
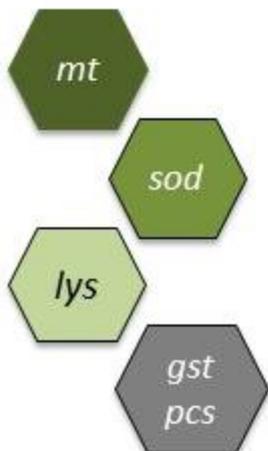
Cd accumulation



4 weeks Cd bioaccumulation



Gene expression



Cd detoxication

